THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 4, pp. 2386–2396, January 22, 2010
© 2010 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

Characterization of a New β(1–3)-Glucan Branching Activity of *Aspergillus fumigatus*

Received for publication, October 21, 2009, and in revised form, November 24, 2009 Published, JBC Papers in Press, November 30, 2009, DOI 10.1074/jbc.M109.077545

Amandine Gastebois[‡], Isabelle Mouyna[‡], Catherine Simenel[§], Cécile Clavaud[‡], Bernadette Coddeville[¶], Muriel Delepierre[§], Jean-Paul Latgé[‡], and Thierry Fontaine[‡1]

From the [‡]Unité des Aspergillus, Institut Pasteur, 25 rue du Docteur Roux, 75015 Paris, the [§]Unité de Résonance Magnétique Nucléaire des Biomolécules, CNRS URA 2185, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris Cedex 15, and the [¶]Laboratoire de Glycobiologie Structurale et Fonctionnelle, UMR 8576 CNRS, Université des Sciences et Technologies de Lille Flandres-Artois, 59655 Villeneuve d'Ascq Cedex, France

A new HPLC method was developed to separate linear from β(1–6)-branched β(1–3)-glucooligosaccharides. This methodology has permitted the isolation of the first fungal β(1–6)/β(1–3)-glucan branching transglycosidase using a cell wall autolysate of *Aspergillus fumigatus* (Af). The encoding gene, Af*BGT2* is an ortholog of Af*BGT1*, another transglycosidase of *A. fumigatus* previously analyzed (Mouyna, I., Hartland, R. P., Fontaine, T., Diaquin, M., Simenel, C., Delepierre, M., Henrissat, B., and Latgé, J. P. (1998) *Microbiology* 144, 3171–3180). Both enzymes release laminaribiose from the reducing end of a β(1–3)-linked oligosaccharide and transfer the remaining chain to another molecule of the original substrate. The AfBgt1p transfer occurs at C-6 of the non-reducing end group of the acceptor, creating a kinked β(1–3;1–6) linear molecule. The AfBgt2p transfer takes place at the C-6 of an internal group of the acceptor, resulting in a β(1–3)-linked product with a β(1–6)-linked side branch. The single Af*bgt2* mutant and the double Af*bgt1*/Af*bgt2* mutant in *A. fumigatus* did not display any cell wall phenotype showing that these activities were not responsible for the construction of the branched β(1–3)-glucans of the cell wall.

The fungal cell wall is a highly dynamic essential organelle that protects the organism from external stress and accounts for 20 to 40% of the cellular dry weight (1). It is a rigid but permeable structure mainly composed of fibrillar (β-glucans, chitin) or amorphous (α-glucans, mannans) polysaccharides. The central core of the *Aspergillus fumigatus* cell wall is composed of β(1–3)/β(1–6)-branched glucan chains on which other polysaccharides (chitin and galactomannan, β(1–3)/(1–4)-glucan) are cross-linked (2). The chitin-β(1–6)-branched β(1–3)-glucan complex is found in almost all yeast and filamentous fungi and is thought to be responsible for cell wall stability.

β(1–3)-Glucans are synthesized by a plasma membrane-bound glucan synthase complex, which uses UDP-glucose as a substrate and extrudes linear chains through the membrane (3). Once these glucans reach the cell wall space, they have to become β(1–6) branched before they can serve as an anchor for other polysaccharides.

The first known β(1–3)-glucanosyltransferase family of *A. fumigatus* (Gelp) splits a β(1–3)-glucan chain internally and transfers the newly generated reducing end to the non-reducing end of another β(1–3)-glucan molecule through a β(1–3) linkage, resulting in elongation of the glucan chain (4). Gelps are glycosylphosphatidylinositol-anchored proteins that have orthologs in yeasts (Gasp in *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*, Phrp in *Candida albicans* (5–7)) and filamentous fungi to form the family GH72 of glycoside hydrolase in the carbohydrate active enzyme data base (CAZy). These specific β(1–3)-glucanosyltransferase activities play an important role in the biosynthesis of the fungal cell wall (5, 7–9), showing that remodeling of β(1–3)-glucan chains are essential in cell wall organization.

The second family of β(1–3)-glucanosyltransferases contains AfBgt1p of *A. fumigatus* and Bgl2p of *S. cerevisiae*. These glucanosyltransferases catalyze the release of a laminaribiose unit from the reducing end of a substrate and transfer the newly generated reducing end to the non-reducing end of another β(1–3)-glucan molecule, generating a new β(1–6) linkage (10, 11). Both null mutants display a phenotype identical of the parental strain.

None of these transglycosidase activities described to date are responsible for the synthesis of a branched β(1–3)-glucan. Here we report a joint molecular and biochemical approach that led to the discovery of the first β(1–3)-glucan branching activity in fungi.

MATERIALS AND METHODS

Strains, Plasmids, and Growth Conditions—The *A. fumigatus Afbgt1* mutant, obtained from the parental strain CBS144.89 (11), was used for enzyme purification and the CEA17*ku80*Δ strain (12) was used for gene deletion. They were maintained in 2% malt agar slants at room temperature. Malt agar slants were supplemented with 150 μg/ml of hygromycin B (Sigma) for the *Afbgt1* and *Afbgt2* mutants and supplemented with 150 μg/ml of hygromycin B and 20 μg/ml of phleomycin (Invivogen) for the *Afbgt2*Af*bgt1* mutant. Cultures grown in Sabouraud liquid medium (2% glucose + 1% mycopeptone, Biokar Diagnostics, Pantin, France) were used for DNA extraction. For transformation experiments, minimal medium (glucose, 10 g/liter; ammonium tartrate, 0,92 g/liter; KCl, 0.52 g/liter; $MgSO_4 \cdot 7H_2O$, 0.52 g/liter; KH_2PO_4, 1.52 g/liter; trace element solution (13), 1 ml/liter, pH adjusted to 6.8) was used.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1–S5.

[1] To whom correspondence should be addressed. Tel.: 33-1-45-68-82-25; Fax: 33-1-40-61-34-19; E-mail: tfontain@pasteur.fr.

Drug inhibition assays were performed in 1% yeast extract medium (9). The *Escherichia coli* strain that was used to produce the recombinant endo-β(1–3)-glucanase (laminarinase A) from *Thermotoga neapolitana* was a kind gift from Dr. Vladimir V. Zverlov, Institute of Molecular Genetics, Russian Academy of Science, Moscow, Russian Federation) (14).

Preparation of Cell Walls and Autolysis—The *Afbgt1* mutant was grown in a 4-liter fermenter in 2% glucose and 1% mycopeptone for 24 h at 25 °C as described previously (4). The mycelium cell wall was prepared as described (4). The cell wall was resuspended in 100 mM sodium acetate, pH 6.2, containing 5 mM sodium azide (around 250 g, wet weight/liter) and incubated at 37 °C under agitation for 3 h. The suspension was centrifuged (10,000 × *g*, 10 min) and the supernatant was dialyzed against 20 mM Tris-HCl, pH 7.5.

Protein Purification—The dialyzed autolysate was applied to a DEAE-Sepharose column (10 × 3.2 cm, Sigma) equilibrated in 20 mM Tris-HCl, pH 7.5, and eluted with a linear gradient of 0–0.5 M NaCl (400 ml) at a flow rate of 25 ml/h. Fractions containing branching activity were pooled, dialyzed against 20 mM Tris-HCl, pH 8.0, applied to a DEAE-5PW column (7.5 × 0.8 cm, TosoHaas), and eluted with a NaCl gradient (0–250 mM in 50 min and 250–500 mM in 10 min) at the flow rate of 0.75 ml/min. Fractions containing branching activity were pooled, dialyzed against 20 mM sodium acetate, pH 4.0, applied to a Mono-S column (HR 5/5, GE Healthcare), and eluted with a NaCl gradient (0–250 mM in 50 min and 250–500 mM in 10 min) at the flow rate of 0.75 ml/min. Fractions containing branching activity were pooled and dialyzed against 20 mM NaOAc, pH 5.0, and stored at −20 °C.

SDS-PAGE and Western Blot—The protein amount was estimated by the Bradford colorimetric assay (Bio-Rad) using bovine serum albumin as standard. Protein samples were analyzed by SDS-PAGE using 10 or 12% acrylamide separating gels (15). Protein bands were visualized by silver nitrate or Coomassie Blue staining. Galactofuranose containing glycoproteins were detected by Western blot with a monoclonal antibody (E2b (16)).

Deglycosylation Methods—Enzymatic *N*-deglycosylation of purified protein was carried out using recombinant PNGase F (Roche Applied Science) according to the manufacturer's instructions. Total chemical deglycosylation was carried out using the trifluoromethanesulfonic acid (TFMS)[2] reagent (17). 10 μg of purified protein was freeze-dried and kept under vacuum in the presence of P_2O_5. Sample was treated with 50 μl of TFMS/anisol solution (2/1, v/v) in a ice-water bath under argon atmosphere for 3 h. The reaction was stopped by addition of 60% pyridine in an ethanol/dry ice bath until pH 6 was achieved. Deglycosylated protein samples were dialyzed against water and analyzed by SDS-PAGE.

Peptide Sequencing—Internal peptides were obtained after in-gel endolysin C digestion of the proteins that had been separated by SDS-PAGE. N-terminal and internal peptide sequencing was performed by J. d'Alayer (Laboratoire de microséquençage des protéines, Institut Pasteur, Paris) on an Applied Biosystems 470 gas-phase sequencer, as previously described (18).

Production and Characterization of Linear and Branched β(1–3)-Glucan Oligosaccharides—Branching activity was based on the detection of the endo-β(1–3)-glucanase-resistant branch point by HPLC. A mixture of linear β(1–3)-glucan oligosaccharides, used as substrate for branching activity, was obtained by acetolysis of curdlan (a kind gift from Hidemitsu Kobayashi) as previously described without the BH_4Na reduction step (19). Matrix-assisted desorption ionization-time of flight (MALDI-TOF) analysis showed that the oligosaccharide fraction used is a mixture of DP5 to DP20 with an estimated average DP of 10. Soluble β(1–3)-glucans were freeze-dried and stored at room temperature. Individual reduced laminarioligosaccharides were obtained by acetolysis of curdlan and reduced by 10 mg/ml of BH_4Na in 0.1 M NaOH overnight at room temperature (19), then purified by high performance anion exchange chromatography (HPAEC) as described below.

Analysis of the Laminarinase-resistant Product (G3)—β(1–6)-Branched β(1–3)-glucan oligosaccharides were previously purified as the SNQz I-B fraction (2). 15 mg of SNQz I-B fraction was digested by 500 μl of recombinant laminarinase A (Lam-A, specific activity: 10 μmol of equivalent glucose/ml/min) in 1 ml of 100 mM sodium acetate, pH 6.2, at 45 °C for 24 h (14). The resulting products were purified by gel filtration on a Bio-Gel P2 column (Bio-Rad, 1.4 × 90 cm) equilibrated in 0.2% acetic acid at the flow rate of 5 ml/h. Sugars were detected by refractometry and the laminarinase A-resistant product (G3) was identified by HPAEC as described below. The size of G3 was determined by MALDI-TOF. Methylation analysis was done on the borodeuteride-reduced G3 using the NaOH procedure (20). Permethylated sample was analyzed by GC-MS after trifluoroacetic acid hydrolysis, reduction, and peracetylation.

Enzymatic Assays—β(1–3)-Glucanase activity was measured by reducing sugar assay using the *p*-hydroxybenzoic acid hydrazide reagent with borohydride-reduced laminarin as previously described (4).

The presence of branching activity was assayed by incubating enzyme fractions in 50 mM sodium acetate, pH 5, at 37 °C with soluble non-reduced linear β(1–3)-glucans (4 mg/ml final concentration) in a final volume of 40 μl for 2 h. The reaction was stopped by addition of 80 μl of a solution of chloroform/methanol (1/1, v/v). After drying under vacuum, the material was solubilized in 30 μl of 50 mM sodium acetate, pH 6.2, and 10 μl of a Lam-A (specific activity: 10 μmol of equivalent glucose/ml/min) (14). After an overnight incubation at 45 °C, digested products were analyzed by HPAEC as described below.

Transfer Product Analysis—To characterize transfer products, reduced laminarioligosaccharides (rG5, rG6, rG7, rG8, rG10, and rG12) were incubated at 3 mM final concentration with 0.05 μg of recombinant enzyme in 30 μl of 50 mM sodium acetate, pH 5.5, at 37 °C. Enzymatic products were analyzed by HPAEC. For a complete characterization of products, 6 mg of reduced laminarihexaose (rG6) was incubated in the same con-

[2] The abbreviations used are: TFMS, trifluoromethanesulfonic acid; DP, degree of polymerization; GC, gas liquid chromatography; HPAEC, high performance anion exchange chromatography; HPLC, high pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; ROESY, rotating-frame Overhauser enhancement spectroscopy; Lam-A, laminarinase A.

ditions for 2 h. Transfer products were purified by gel filtration on a TSK-HW40S column (TosoHaas, 90 × 1.6 cm) equilibrated in 0.25% acetic acid at the flow rate of 0.4 ml/min. Oligosaccharides were detected by a refractometric detector. Separated fractions were freeze-dried and analyzed by MALDI-TOF, methylation, and NMR. Methylation was performed with the NaOH procedure (20).

HPAEC (Dionex, Model ISC3000) of Oligosaccharides—The Lam-A digests were analyzed on a CarboPAC-PA1 column (4.6 × 250 mm) using NaOH (50 mM) and sodium acetate (500 mM) in 50 mM NaOH as eluent A and B, respectively. The column was pre-equilibrated for 20 min in 98%A + 2%B. Following sample injection, a gradient run (flow rate 1 ml/min) was performed as follows: 0–2 min, isocratic step (95% A + 2% B), 2–15 min 98% A + 2% B − 80% A + 20% B; 15–22 min 80% A + 20% B − 57% A + 43% B; 22–23 min 57% A + 43% B − 100% B; and 23–25 min 100% B. Samples were detected on a pulsed electrochemical detector. Transfer products obtained after enzymatic reaction were analyzed by HPAEC on a CarboPAC-PA200 column (3.2 × 250 mm). The same eluents were used in the following condition, a flow rate of 0.35 ml/min and a gradient run: 0–2 min, isocratic step (95% A + 2% B), 2–15 min, 98% A + 2% B − 65% A + 35% B; 15–35 min, 65% A + 35% B − 40% A + 60% B; 35–37 min, 40% A + 60% B − 100% B; and 37–40 min, 100% B. Reduced laminarioligosaccharides were purified on a semipreparative CarboPAC-PA1 column (9 × 250 mm). The same eluents are used in the following conditions: flow rate of 4 ml/min and a gradient run: 0–2 min, isocratic step (90% A + 10% B); 2–20 min, 90% A + 10% B − 60% A + 40% B; 20–45 min, 60% A + 40% B − 40% A + 60% B; 45–50 min, 40% A + 60% B − 100% B; and 50–51 min, 100% B.

Gas Liquid Chromatography (GC) and Mass Spectrometry (MS)—GC and GC-MS was performed on a Perichrom PR2100 instrument and on an Automass II apparatus (Finigan) coupled to a CarloErba gas chromatograph (model 8000top) as previously described (21). MALDI-TOF mass spectra were acquired on a Voyager Elite DE-STR mass spectrometer (Perspective Biosystems, Framingham, MA) equipped with a pulsed nitrogen laser (337 nm) and a gridless delayed extraction ion source. The spectrometer was operated in positive reflectron mode as previously described (22).

NMR Spectroscopy—NMR spectra were acquired at 288 K on a Varian Inova 600 spectrometer equipped with a cryogenically cooled triple resonance $^{1}H\{^{13}C/^{15}N\}$ pulsed field gradient probe. Samples were dissolved in 420 μl of D_2O (99.97% ^{2}H atoms, Euriso-top, CEA, Saclay, France) and transferred in a 5-mm Shigemi tube (Shigemi Inc., Allison Park, PA). ^{1}H and ^{13}C chemical shifts were referenced to external 2.2-dimethyl-2-silapentane-5-sulfonate sodium salt (23). Intraglycosidic residue nuclei were first assigned as previously described (2). Then, $^{1}H,^{1}H$ coupling constants were extracted from one- and/or two-dimensional spectra (^{1}H resolution of 0.1 and 0.6 Hz, respectively). The anomeric configuration was established from knowledge of the $^{3}J_{1,2}$ value. Finally, interglycosidic linkages were established via through-space dipolar interactions using a ^{1}H-^{1}H ROESY experiment (mixing time of 250 ms) (24) and/or via multiple bond ^{1}H-^{13}C correlations using a ^{1}H-^{13}C gradient-selected heteronuclear multiple bond correlation experiment (long range delay of 60 ms) (25).

Enzymatic Kinetic Analysis—The amount of transfer product was measured by the integration of the branched glucan area analyzed by HPAEC after Lam-A digestion of the Bgt2p reaction products. The pH optimum was studied by incubation of 0.2 μg of recombinant protein with 20 mg/ml of laminarioligosaccharides in 40 μl of 125 mM sodium citrate buffer in pH range 3 to 9 at 37 °C for 30 min. Temperature optimum was estimated by incubation of 0.2 μg of recombinant protein with 20 mg/ml of laminarioligosaccharides in 40 μl of 0.2 M sodium acetate buffer, pH 5.5, in the temperature range of 4 to 65 °C for 15 min. Specific activities and K_m were estimated by incubation of 0.075 μg of recombinant protein in 0.2 M sodium acetate buffer, pH 5.5, at 37 °C for 30 min with a substrate range concentration of 1 to 12 mg/ml.

Construction of the AfBGT2 Deletion Cassette—The cassettes for deleting Af*BGT2* and Af*BGT1* genes were constructed by PCR fusion with the method described by Lamarre *et al.* (26). The resistance marker used to replace Af*BGT2* was the *HPH* gene of *E. coli* coding for hygromycin B phosphotransferase obtained from the pAN7-1 plasmid (27). Site-specific recombination was ensured by association of ~1.4-kb upstream and 1.3-kb downstream Af*BGT2* flanking fragments. In a first PCR round (supplemental Fig. S1) flanking region 1 (amplicon 1, primers Bgt2A-Bgt2B), flanking region 2 (amplicon 2, primers Bgt2E-Bgt2F), and the *HPH* gene (amplicon 3, primers Bgt2C-Bgt2D) were amplified, respectively, from wild type DNA template and pAN7.1 with the primers cited in supplemental Table S1. Thirty cycles consisting of a 30-s 95 °C melting step and a 3-min 68 °C annealing/extension step were performed (Advantage 2 polymerase, Clontech). Primers B, C, D, and E were 60-bp chimeric oligonucleotides, containing at the 5′-end a reverse complement sequence (B with C and D with E) for fusion PCR. The obtained PCR products were gel-purified and used for a second PCR step that allowed fusion of these three separate fragments by using Bgt2A and Bgt2F primers. The PCR conditions were the same as described above except at a 6-min elongation step. The resulting PCR was purified and used to transform *A. fumigatus* conidia.

The *BLE* gene, coding for phleomycin resistance, was used as a resistance marker and amplified from the pAN8-1 to delete the Af*BGT1* gene (28). Site-specific recombination was ensured by association of an ~1-kb upstream and 1.1-kb downstream *BGT1* flanking fragment. Primers Bgt1A and Bgt1B were used to amplify the first flanking region, primers Bgt1E and Bgt1F to amplify the second flanking region, and primers Bgt1C and Bgt1D to amplify thd *BLE* gene. To perform the PCR fusion step primers Bgt1A and Bgt1F were used.

AfBgt2 and Double Afbgt1/Afbgt2 Mutants—The fusion PCR product (1 to 2 μg) was used to transform *A. fumigatus* CEA17*ku80*Δ conidia by the electroporation method described by Sanchez and Aguirre (29) with subsequent modifications. Conidia from the CEA17*ku80*Δ strain were washed five times with water and 10^9 conidia were inoculated in 125 ml of YG medium (0.5% yeast extract, 2% glucose) and incubated at 37 °C at 300 rpm for 4 h. Conidia were recovered by centrifugation, washed with water, inoculated in 12.5 ml of YG medium (1%

yeast extract, 1% glucose, 20 mM Hepes, pH 8.0) and incubated for 1 h at 30 °C at 100 rpm. Conidia were centrifuged and resuspended in 1 ml of cold 1 M sorbitol. One to 2 μg of DNA was added to 50 μl of conidial suspension, incubated for 15 min on ice, and transferred to 0.1-cm electroporation cuvettes. Electroporation was performed using the Bio-Rad gene pulser (Gene Pulser Xcell) with the following parameters: voltage, 1 kV; capacitance, 25 microfarads; and resistance, 400 Ω. After transformation, 1 ml of cold YG medium was added to the cuvette, conidia were transferred to a 10-ml sterile tube and incubated on ice for 15 min. Tubes were incubated at 30 °C at 100 rpm for 1 h and 30 min. Conidia were plated on minimal medium (500 μl/9-cm diameter Petri dish) and incubated at 20 °C overnight. Hygromycin (150 μg/ml) or phleomycin (20 μg/ml) were added in a 10-ml overlay of minimal medium + 0.7% agarose, to allow transformants selection, and plates were incubated at 37 °C for 2 days.

Genomic DNAs from hygromycin- or phleomycin-resistant transformants and the parental strain were isolated as described by Girardin *et al.* (30). To check integration of the *HPH* cassette at the Af*BGT2* locus, DNA of the CEA17*ku80*Δ and Af*bgt2* mutant strains were digested with XbaI and HindIII restriction enzymes (Roche Applied Science) and verified by Southern blot analysis with probes corresponding to the second flanking region. To analyze integration of the *BLE* cassette at the Af*BGT1* locus in the Af*bgt2* mutant, DNA of CEA17*ku80*Δ and the transformants were digested by XbaI and HindIII to check the Af*BGT2* interruption and HindIII/EcoRI to check Af*BGT1* interruption and verified by Southern blot analysis with a PCR product corresponding to the second flanking region as probe.

Phenotypic Analysis—Conidial germination and mycelial growth were analyzed on medium of a different composition (1% yeast extract + 3% glucose medium, Sabouraud, minimal medium, and RPMI medium) in 2% agar or liquid medium, at 20, 30, 37, or 50 °C, in the absence or presence of sorbitol (0.5 to 2 M), at different pH values (5, 7, and 9). The effect of the drugs tested was as described previously (9).

Cell Wall Analysis—After 24 h growth at 37 °C in Sabouraud medium, mycelium of wild type and the *Afbgt2* mutant strain were filtered on a Buchner funnel and extensively washed with water. Mycelium was broken during 2 min with 1-mm diameter glass beads and 200 mM Tris-HCl, pH 8, in a 50-ml Falcon vial with a Fast-prep apparatus (MP). After protein extraction (2% SDS, 40 mM β-mercaptoethanol, 50 mM Tris-HCl, 5 mM EDTA, pH 7.4, 100 °C for 10 min), alkali-soluble and insoluble fractions of the cell wall were prepared as previously described (11). Neutral hexoses and hexosamines from the cell wall were estimated by phenol/sulfuric acid and dimethylaminobenzaldehyde colorimetric assays, respectively (31, 32). Monosaccharides were identified by GC as alditol acetates obtained after hydrolysis (4 N trifluoroacetic acid, 100 °C, 4 h, or 8 N HCl, 3 h, 100 °C) (33). Branching of cell wall β(1–3)-glucan was estimated by HPAEC after laminarinase A digestion of the alkali-insoluble fraction. 50 μg of alkali-insoluble fraction were digested by Lam-A (10 μl at 10 μmol/min/ml) in 100 μl of 50 mM sodium acetate, pH 6.2, and incubated for 24 h at 45 °C. The β(1–3)-glucan amount was estimated by Lam-A digestion and the *p*-hydroxybenzoic acid hydrazide reagent for detection (4).

Recombinant Protein Expression and Purification—The yeast *Pichia pastoris* GS115 and the pHILS1 plasmid (Invitrogen) were used to produce the recombinant protein, Bgt2p. A truncated form of Bgt2p lacking 23 amino acids at the C-terminal part (glycosylphosphatidylinositol anchoring sequence) was obtained by PCR amplification of the Af*BGT2* cDNA with primers Bgt2p1 and Bgt2p2 (supplemental Table S1). The Bgt2p1 primer, complementary to nucleotides 56 to 73, contains an XhoI restriction site used for the cloning procedure in the pHILS1 plasmid, and the Bgt2p2 primer, complementary to 1315 to 1333 carries a BamHI site and an in-frame TGA stop codon (supplemental Table S1). The 1286 bp product was cloned in the pHilS1 plasmid, carrying the *HIS* auxotrophy marker, at the XhoI-BamHI site and used to transform the GS115 strain with a lithium chloride method (Invitrogen). Transformants were selected on histidine-depleted medium. The clones carrying integration in the alcohol oxidase gene (*AOX1*) were selected for their inability to grow in methanol medium.

P. pastoris strain was inoculated in BMMY medium (Invitrogen) for 72 h at 30 °C and induction was done by addition of 1% of methanol to the culture medium every 24 h per the manufacturer's instructions (Invitrogen). Five ml of culture supernatant of *P. pastoris* producing Bgt2p were dialyzed against 20 mM Tris-HCl, pH 7.5, then applied to a DEAE 5PW column and eluted with a NaCl gradient (0–250 mM in 50 min and 250–500 mM in 10 min) at the flow rate of 0.75 ml/min. Proteins were detected with a UV detector (λ = 280 nm). The purity of the protein was checked by SDS-PAGE. The protein was dialyzed against 10 mM sodium acetate, pH 6, and kept frozen.

RESULTS

An HPLC Method to Detect β(1–6)-Branching of β(1–3)-Glucan Chains—The digestion of linear β(1–3)-glucan oligosaccharides by a recombinant endo β(1–3)-glucanase (14) produced glucose and laminaribiose (G2). In contrast, the enzymatic digestion of a β(1–6)-branched β(1–3)-glucan fraction from the alkali-insoluble cell wall extract produced an additional peak (G3), eluted after the laminaribiose by HPAEC (Fig. 1). MALDI-TOF spectra revealed one $[M + Na]^+$ pseudomolecular ion at $m/z = 527$, corresponding to a trisaccharide. Methylation analysis done on the borodeuteride reduced G3 revealed the presence of three equimolar methyl-ethers: 1,2,4,5,6-penta-*O*-methyl-glucitol, 2,3,4,6-tetra-*O*-methyl-glucitol, and 2,3,4-tri-*O*-methyl-glucitol corresponding to a glucose at the reducing end substituted at position 3, a terminal non-reducing end glucose and a glucose substituted in position 6, respectively (Fig. 1*C*). Based on these MALDI-TOF and methylation data, the G3 structure was deduced as the following linear sequence βGlc1–6βGlc1–3GlcOH. This HPAEC detection of the β(1–3)/(1–6)-glucan was used to develop an HPLC assay to detect β(1–3)-glucan branching activity.

Purification of a β(1–3)-Glucan Branching Enzyme—A cell wall autolysate of a Af*bgt1* mutant (11) was incubated with a curdlan acetolysate containing a mixture of linear soluble β(1–3)-glucan chains with DP 5–20. The presence of G3 after lami-

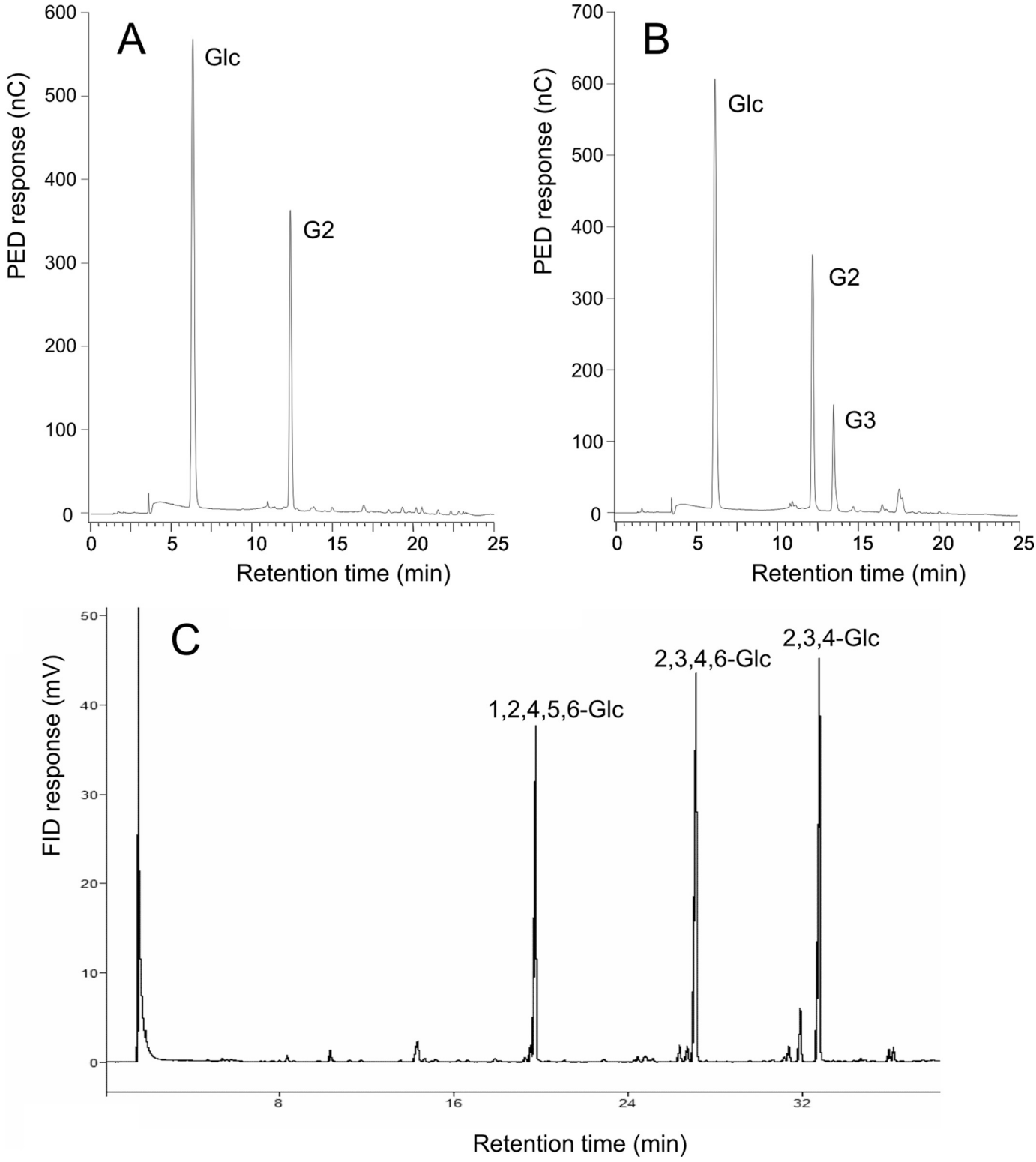

FIGURE 1. **Analysis of Lam-A digestion of β(1–3)-glucans.** HPAEC on a CarboPAC-PA1 of the Lam-A digest of linear laminarioligosaccharides (*A*) and β(1–6)-branched β(1–3)-glucan (SNQz IB fraction) (*B*). GC analysis of partially methylated alditol acetates obtained after permethylation of borodeuteride-reduced G3 (*C*). *Glc*, glucose; *G2*, laminaribiose; *1,2,4,5,6-Glc*, 1,2,4,5,6-penta-*O*-methyl-3-*O*-acetylglucitol; *2,3,4,6-Glc*, 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetylglucitol; *2,3,4-Glc*, 2,3,4-tri-*O*-methyl-1,5,6-tri-*O*-acetylglucitol.

narinase A digestion and the HPLC step indicated the presence of a β(1–3)-glucan branching enzyme in the cell wall autolysate.

Two fractions containing a β(1–3)-glucan-branching activity were eluted at 40 and 100 mM NaCl, respectively, in a DEAE-5PW column (Fig. 2). After the chromatographic step on the MonoS column, SDS-PAGE showed that the first fraction migrated as a wide band with a molecular mass of 25 to 50 kDa typical of the glycosylated protein, and the second fraction as a

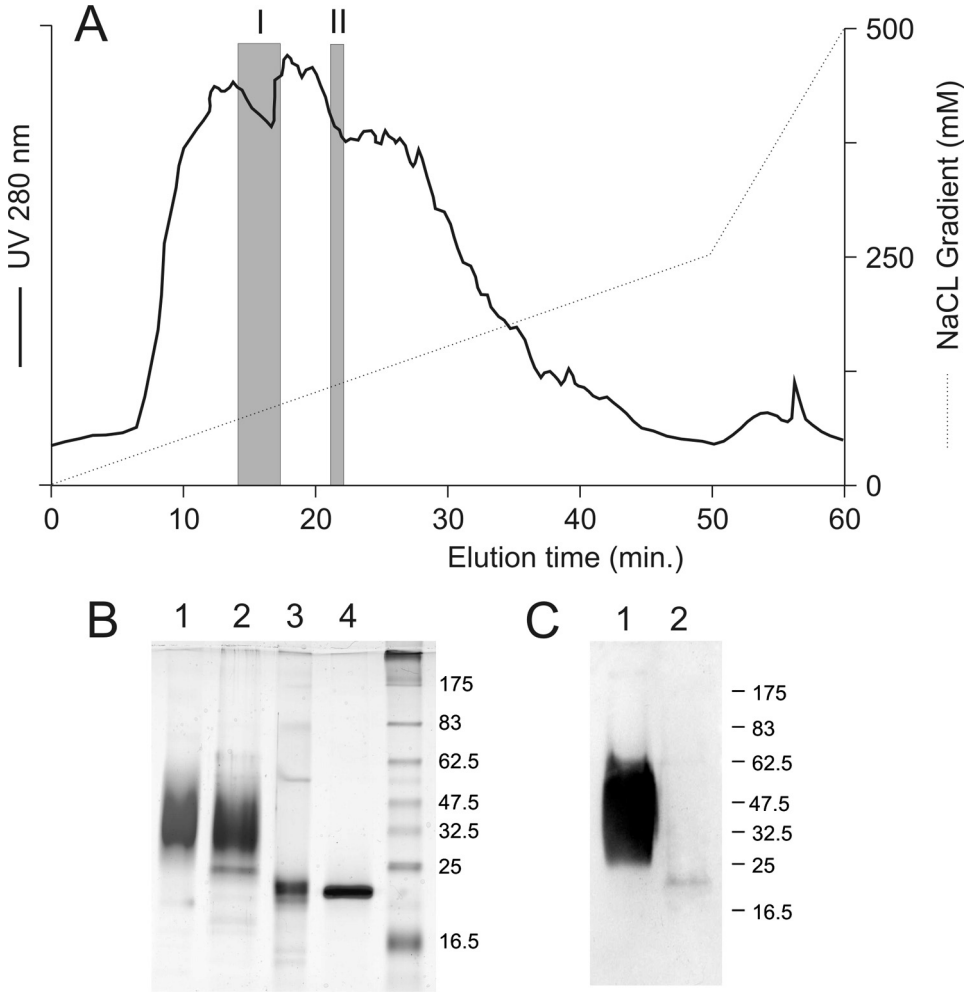

FIGURE 2. **SDS-PAGE and Western blot analysis of Bgt2p from *A. fumigatus*.** *A*, chromatographic purification step on the DEAE-5PW column of the β(1–3)-glucan branching activity from the cell wall autolysate. *B*, SDS-PAGE analysis and silver nitrate staining. *Lane 1*, purified fraction I; *lane 2*, PNGase-treated fraction I; *lane 3*, TFMS-treated fraction I; *lane 4*, purified fraction II; *C*, Western blot and detection with anti-galactofuranose monoclonal antibody; *lane 1*, purified fraction I; *lane 2*, purified fraction II.

discrete band with a molecular mass of 21 kDa (Fig. 2). Fraction I was digested with *N*-glycosidase F or treated with TFMS reagent. *N*-Glycosidase F-treated enzyme did not modify the SDS-PAGE pattern. In contrast, TFMS treatment released a protein with a molecular mass of 21 kDa, indicating that the native protein of fraction I was highly *O*-glycosylated. The presence of mannose and galactose residues identified by GC in the 1/1.2 respective ratio (not shown), and the positive reactivity in the Western blot of fraction I with anti-galactofuran monoclonal antibody indicated the presence of *O*-glycan with galactofuranose residues (Fig. 2). This was in agreement with the presence of *O*-glycan previously characterized in glycoproteins isolated from *A. fumigatus* mycelium (34).

Analysis of the AfBGT2 Gene Sequence—The sequence of three peptides from fraction I (AAIAQYGDDLAK, NLVGTSGFTSAR, and ANAGIGTNPDEI) and the N-terminal peptide (AHQGFN) from fraction II were obtained. Based on these sequences, the homology search in the *A. fumigatus* JCVI genome sequence showed that all four peptides belong to the same protein encoded by a gene we named Af*BGT2* (AFUA_3G00270) (supplemental Fig. S2). The 1341-bp long open reading frame predicted a 447-amino acid protein with a predicted molecular mass of 44.6 kDa. This gene contains one intron, four putative *N*-glycosylation sites, a region rich in serine at the C terminus (14%), a hydrophobic N terminus, and a C terminus characteristic of glycosylphosphatidylinositol-anchored proteins with a carboxyl peptidase cleavage site consensus sequence (Ω site: Asn^{423}) (supplemental Fig. S2). Comparative genomic analysis revealed that AfBgt2p orthologs only shared homology with members of glycosyl hydrolases family 17 (CAZy) and was homologous to AfBgt1p (17% identity) and ScBgl2p (18% identity). The GH17 family is constituted of enzymes found in plant and fungi only, and are characterized by a catalytic site composed of two glutamic acids identified in plant enzymes (35, 36). Peptide sequence alignment between AfBgt1p and *S. cerevisiae* Bgl2p suggested that the two glutamic acids of the catalytic site were conserved and were Glu^{128} and Glu^{239} in Bgt2p (supplemental Fig. S2). A recombinant protein, lacking the glycosylphosphatidylinositol signal sequence, was produced in *P. pastoris*. The recombinant protein, rBgt2p, migrated with a molecular mass of 175 kDa (supplemental Fig. S3). After *N*-deglycosylation, the protein had an apparent molecular mass of 43 kDa (not shown), corresponding to sequence predictions. Glycosylation of the rBgt2p was higher than the native *A. fumigatus* protein. In contrast to the recombinant rBgt2p, the deglycosylated form of the native protein was smaller than the predicted M_r (Fig. 2), suggesting that the enzyme was partially degraded by proteolysis during its secretion or during cell wall autolysis.

Characterization of the Transglycosidase Activity—The incubation of proteins, purified from the cell wall autolysate and recombinant protein rBgt2p with a mixture of linear laminarioligosaccharides followed by LamA digestion, produced G1, G2, and the β(1–6)-β(1–3) trisaccharide (G3). This result confirmed that the branching activity of Bgt2p was not influenced by its glycosylation. The enzymatic characterization of the activity was performed with the recombinant protein. Fig. 3 shows the products of the enzymatic reaction obtained after incubation of linear reduced β(1–3)-glucan with rBgt2p. For each reduced laminarioligosaccharide, two types of products were obtained. Based on their retention time, one was the reduced laminaribiose (rG2) and the other had an elution time

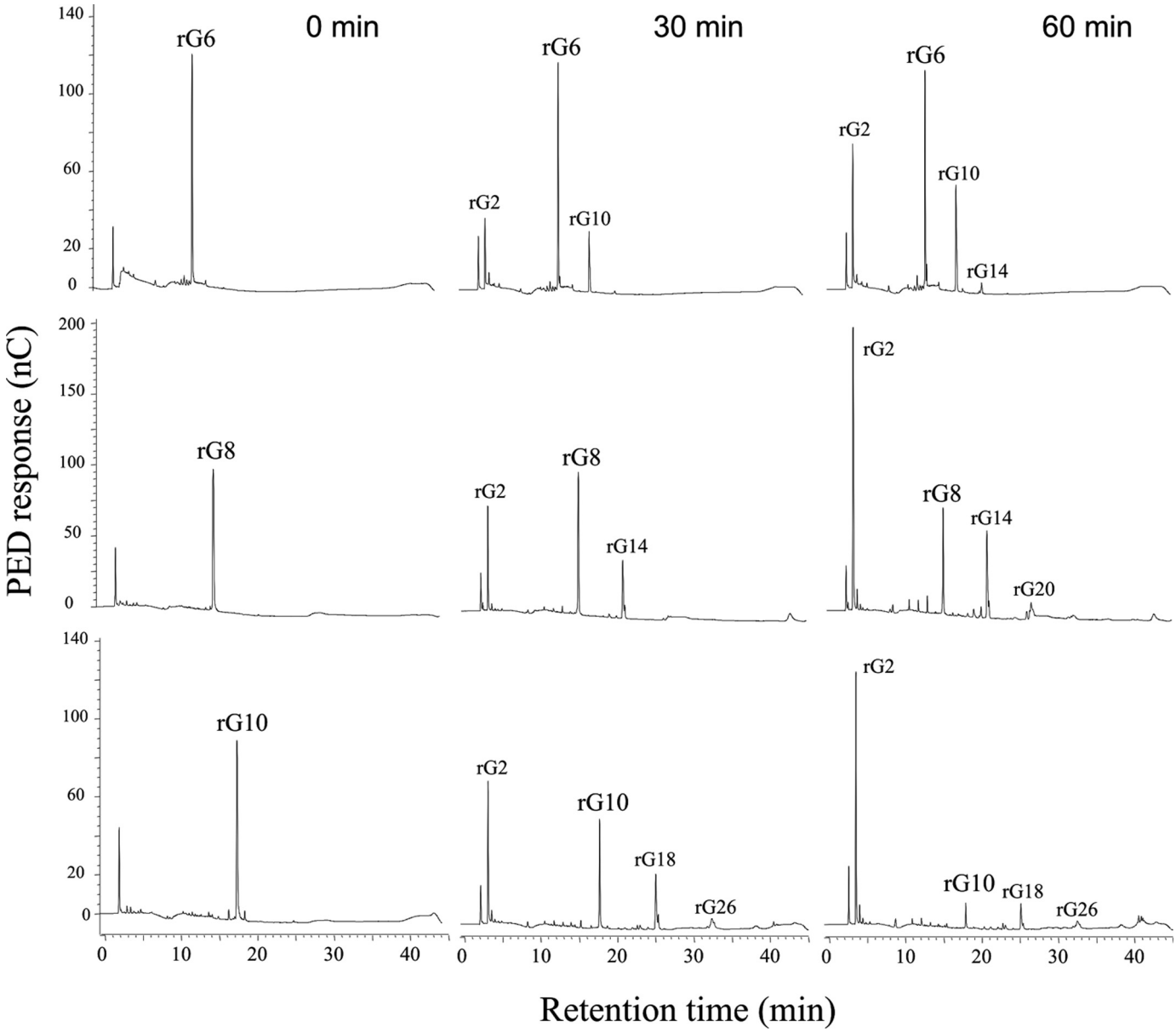

FIGURE 3. **HPAEC on a CarboPAC-PA200 column of products obtained by incubation of reduced laminarioligosaccharides with rBgt2p.** *rGn*, reduced laminarioligosaccharide containing *n* glucose residues.

depending on the substrate size. Using the rG6 as a substrate, MALDI-TOF mass spectra indicated that the higher molecular weight peak corresponded to rG10 $[M + Na]^+$ pseudomolecular ions at $m/z = 2311$. This result indicated a two-step transglycosidase reaction: rG6 + E → E-G4 + rG2 and E-G4 + rG6 → E + rG10. In the first step the enzyme cleaved between glucose units 2 and 3 from the reducing end releasing reduced laminaribiose (rG2) from the reduced substrate. In the second step the enzyme transferred the remainder of the substrate to a second molecule of substrate, used as an acceptor, to give a reduced G10 (rG10).

The methylation analysis of the rG10 transfer product showed the presence of several substitutions of glucose residue (Table 1). In addition to glucose substituted at position 3 and glucose residues from each chain extremity, two other substitutions were observed: substitution at position 6 and bi-substitution at positions 3 and 6. A ratio of 6 between these two types of substitution indicated that the enzyme transferred the glucan donor mainly inside the acceptor chain and for a minor part at the non-reducing end through a 1–6 linkage (Table 1).

The NMR data (chemical shifts and coupling constants) of the rG10 transfer product are summarized in Table 2. The one-dimensional ^1H and two-dimensional ^1H,^{13}C gradient-selected heteronuclear single-quantum correlation spectra exhibited five major signals (4.78/105.2, 4.75/105.3, 4.74/105.4, 4.66/105.7, and 4.53/105.3 ppm) and one minor signal (4.71/105.5 ppm) in the sugar anomeric region. The ^1H and ^{13}C chemical shift analysis of the corresponding sugar spin system and the examination of coupling constant values permitted identification of β-glucose residues. The integration of these 5 major signals in the one-dimensional spectrum showed that they were in the ratio 3/2/2/1/1 (corresponding, respectively, to

A′ + 2A, B′ + B, 2C, E, and G in Table 2 and supplemental Fig. S4). Other ^{1}H signals in the one-dimensional spectrum (4.00 and 4.03 ppm) were not associated with a signal in the anomeric region and were thus assigned to one glucitol residue (supplemental Fig. S4). Downfield shifts were observed for H3 and C3 resonances of A′, A, B′, B, G, and E glucose residues and for the glucitol indicating a 3-O substitution for these residues (in *bold* in Table 2). Furthermore, in the edited gradient-selected heteronuclear single-quantum correlation experiment, four distinct methylene carbons have been identified (3.91-3.73/63.3, 3.77-3.66/64.6, 3.84-3.63/65.4, and 4.20-3.86/71.5 ppm) (data not shown). The downfield shifted C-6 at 71.5 ppm assigned to B and minor D glucose residues indicated that both glucose residues were 6-*O*-substituted (in *bold* in Table 2). The methylene carbons resonating at 64.6 and 65.4 ppm were assigned to the glucitol residue. In agreement with MALDI-TOF analysis, 10 major residues were identified: one glucitol residue, one 3,6-*O*-disubstituted β-Glc (B), six 3-*O*-monosubstituted β-Glc (A′, 2A, B′, E, and G), and two unsubstituted β-Glc residues corresponding to non-reducing ends (2C). These data are indicative of a major 6-*O*-branched β-(1–3)-Glc decasaccharide. In agreement with the two-step enzymatic reaction, it corresponds to a reduced laminarihexaose substituted by a laminaritetraose side chain. The ROESY and gradient-selected heteronuclear multiple bond correlation experiments allowed the establishment of the following laminaritetraose chain sequence: C1–3A1–3B′1–3G, which is branched to the B residue through a β(1–6) linkage. However, position B in the main hexose chain could not be exactly determined. Indeed, due to strong overlap, it was not possible to distinguish between the following two sequences for the main chain: C1–3A1–3B1–3A′1–3E1–3Glcol or C1–3A1–3A′1–3B1–3E1–3Glcol. From these results, the branching point on the main chain occurred on the third or fourth residue from the non-reducing end. A minor 6-*O*-monosubstituted β-Glc residue was identified (D), meaning there was a branching chain on the non-reducing end of the main chain. A total branching degree of 1.0 was determined from the ratio of the -6)-β-Glc- and -3,6)-β-Glc H6′ proton area to the glucitol H2 and H3 protons half-area in the one-dimensional spectrum. From NMR and methylation data, the transglucosidase reaction was estimated at 85% inside the acceptor chain and 15% at the non-reducing end using laminarihexaositol as substrate (Fig. 4). With larger substrates such as rG8 or rG10, transfer products were eluted as doublet or triplet peaks in the HPAEC analysis, indicating a multiple site of transfer on the acceptor (Fig. 3).

In addition to the rG10 transfer product, a rG14 transfer product was observed after a 1-h reaction (Fig. 3). The one-dimensional ^{1}H spectrum of this rG14 was similar to the rG10,

TABLE 1

MALDI-TOF and methylation analyses of transfer product obtained after enzymatic reaction with reduced laminarihexaose

Methyl ethers were analyzed as their itols.

Transfer product		
MALDI-TOF analysis		
m/z [M + Na] $^+$		1663
Corresponding DP		10
Methylation analysis		
Methyl ethers	Linkages	
1,2,4,5,6-Glc	-3Glucitol	0.5
2,3,4,6-Glc	Glc1-	1.6
2,4,6-Glc	-3Glc1-	6
2,3,4-Glc	-6Glc1-	0.2
2,3-Glc	-36Glc1-	1.2

TABLE 2

^{1}H and ^{13}C NMR chemical shifts (ppm) and coupling constants ($J_{H,H'}$ Hz) for the rG10 transglycosidase product

The glucose residues were labeled A to G in order of decreasing chemical shift of their anomeric protons.

	H1, $^3J_{1,2}$, C1	H2, $^3J_{2,3}$, C2	H3, $^3J_{3,4}$, C3	H4, $^3J_{4,5}$, C4	H5, $^3J_{5,6}$, C5	H6, $^2J_{6,6'}$, C6	H6′, $^3J_{5,6'}$
-3)-β-Glc-(1-	4.785	3.535	**3.757**	3.508	#3.5	3.729	3.914
A′	8.9	8.9	#9	#9		#11	
	105.21	75.69	**87.38**	78.28	70.78	63.34	
-3)-β-Glc-(1-	4.783	3.534	**3.767**	3.493	3.493	3.727	3.913
A	8.0	8.9	8.9	#8		#11	
	105.21	75.98	**86.73**	78.28	70.78	63.36	
-3)-β-Glc-(1-	4.756	3.538	**3.775**	3.506	3.506	3.727	3.909
B′	9.4	9.4				#11	
	105.30	75.98 or 75.72	**86.55**	78.20	70.70	63.34	
-3,6)-β-Glc-(1-	4.751	3.558	**3.787**	3.598	3.675	3.881	4.211
B	8.7	8.7	8.9	8.7		10.7	
	105.30	75.97	**86.30**	70.66	77.06	**71.49**	
β-Glc-(1-	4.743	3.336	3.506	3.385	3.465	3.701	3.902
C	7.6	8.6–9.2	9.2	9.6	6.3	12.2	
Nonreducing end	105.42	76.09	78.19	72.19	78.69	63.34	
-6)-β-Glc-(1-	4.712	3.358	3.516	3.475	3.653	3.859	4.202
D	7.62	8.6	8.3	8.7		10.6	
Minor	105.52	75.91	78.01	72.13	77.35	**71.49**	
-3)-β-Glc-(1-	4.660	3.568	**3.768**	3.514	3.492	3.724	3.920
E	7.8	8.4	9.3	#8		11.5	
	105.68	75.94	**86.63**	77.97	70.95	63.33	
-β-Glc-(1–6)-	4.538						
F							
Minor							
-3)-β-Glc-(1–6)-	4.530	3.493	**3.733**	3.489	3.496	3.732	3.907
G	7.6	8.6	9.1	#8		11.7	
	105.27	75.67	**86.74**	78.12	70.72	63.33	
Glucitol	3.771-3.657	4.002	**4.034**	3.627	3.884	3.835-3.627	
	$^3J_{1,2}$ = 4.4			9.1		11.1	
H	$^3J_{1,2'}$ = 6.2						$^2J_{1,1'}$ = 12,1
Reducing end	64.57	75.46	**81.19**	72.66	73.35	65.35	

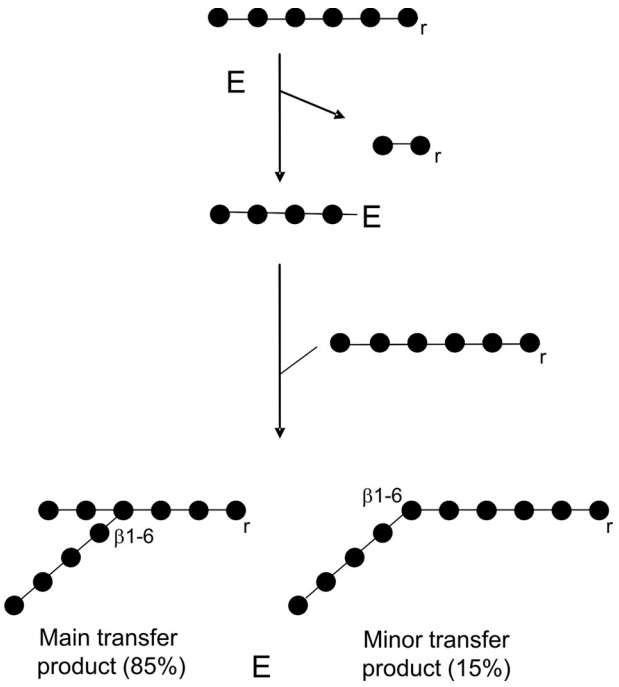

FIGURE 4. **Scheme of the two steps of the enzymatic reaction of rBgt2p with laminarihexaose as substrate.**

except for the intensities of signals, showing an increase in -3,6-*O*-disubstituted and 6-*O*-monosubstituted β-Glc residues relative to glucitol (supplemental Fig. S4). A total branching degree of 1.9 was determined from the ratio of -6)-β-Glc- and -3,6)-β-Glc H6′ proton areas to glucitol H2 and H3 proton half-areas in the one-dimensional spectrum, indicating that the rG10 transfer product was used again as a substrate. This enzyme is a new β(1–3)-glucanosyl transferase producing cross-linking between β(1–3)-glucan chains through a β(1–6) linkage as described below.

The $G_{(2n-2)}$ transfer product has been observed with all substrates tested with a DP ≥ 5 (Figs. 3 and 5). After 60 min of incubation with the DP12 (rG12), the disproportionate amount of rG2, compared with rG18 and rG26, indicated that transfer products were too large to be eluted from the column. The percentage of substrate, first, second, and third transfer products, estimated from pulsed amperometric detection, showed that the kinetic rate of transferase reaction and the recycling of transfer products increased with substrate size (Fig. 5). HPLC analysis of the Lam-A digest of the incubation of rBgt2p with curdlan revealed that rBgt2p was able to branch insoluble β(1–3)-glucan (data not shown).

In addition to transfer products, other smaller peaks were observed suggesting that a hydrolytic activity occurred at the same time. The estimation of these peaks in comparison with transfer products showed that lowering the concentration of substrate promoted hydrolysis (Fig. 6). At a concentration lower than 0.1 mM rG10, less than 50% of the transfer activity was observed, whereas at 1 mM rG10 the transfer activity reaches 90%, indicating that the enzymatic reaction is an equilibrium between hydrolysis and transglucosylation depending on the substrate concentration.

Optimum pH and temperature were estimated to be 5.5 and 45 °C, respectively (data not shown). Using a range of substrate

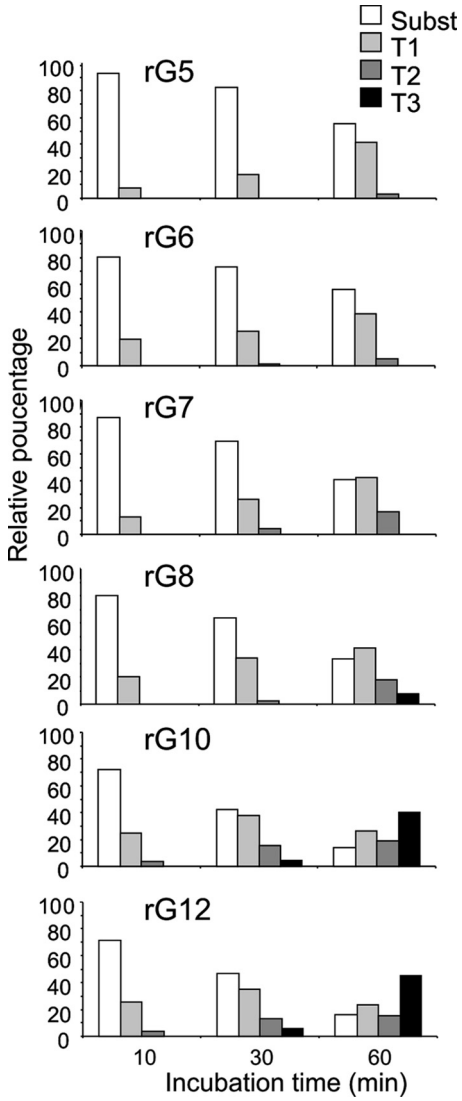

FIGURE 5. **Effect of substrate size on the transfer product formation.** *Subst*, substrate; *T1*, first transfer product; *T2*, second transfer product; *T3*, third transfer product.

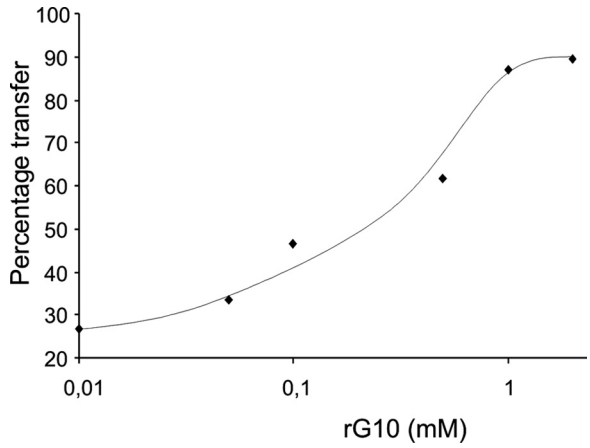

FIGURE 6. **Effect of the substrate concentration.** Transfer rate with different concentrations of reduced laminaridecaose (rG10) used as a substrate. Hydrolyzed and transfer products were estimated by HPAEC on a CarboPAC-PA200 column.

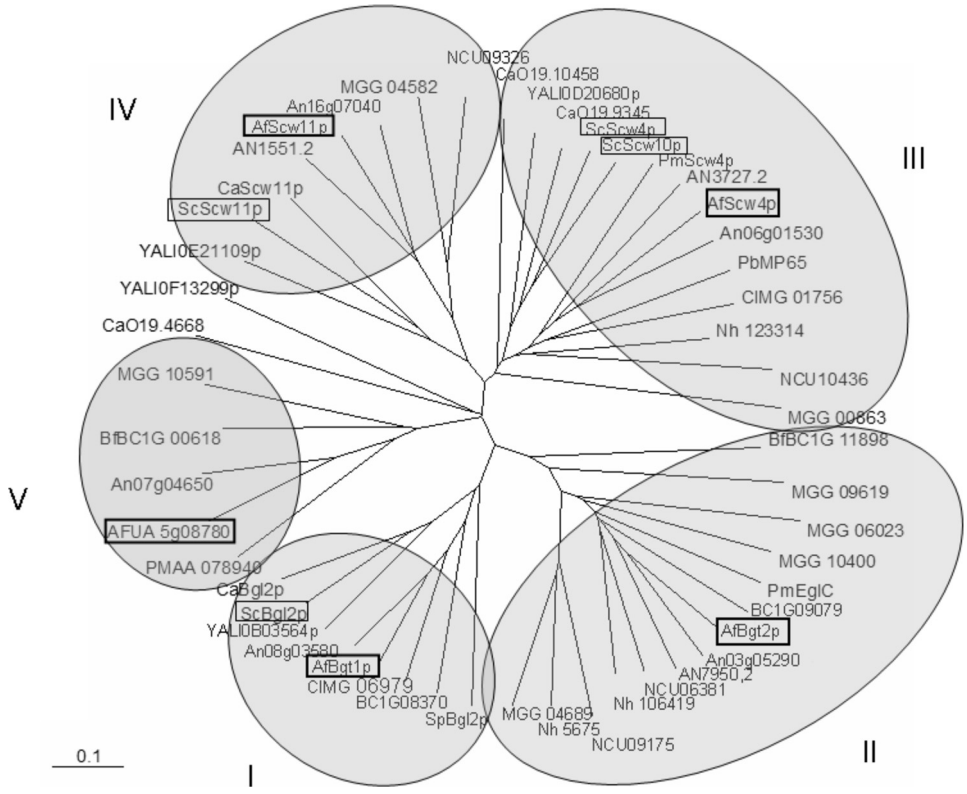

FIGURE 7. **Dendrogram analysis of the GH17 protein family identified in yeast and filamentous fungus.** The sequences of known and putative GH17 family proteins identified in *S. cerevisiae* (*Sc*), *C. albicans* (*Ca*), *Magnaporthe grisea* (MGG), *Schizosacchromyces pombe* (*Sp*), *Aspergillus niger* (*An*), *Aspergillus nidulans* (*AN*), *Neurospora crassa* (*NCU*), *Yarrowia lipolytica* (*YAL*), *Nectria hematoccoca* (*Nh*), *Coccidioides immitis* (*CIMG*), *Penicillium marneffei* (*PMAA* or *Pm*), *Botryotinia fuckeliana* (*BC* or *BfBC*), and *Paracoccidioides brasilensis* (*Pb*) were aligned and the dendrogram was generated using Clustaw X.

(mixture of laminari-oligosacccharides) concentrations of 1 to 12 mg/ml showed that this enzyme activity followed a Michaelis-Menten kinetic with a K_m of 3 mM and a specific activity estimated at 1.2 μmol of G3/min/μg of protein (supplemental Fig. S5). At substrate concentration, the K_m value of the hydrolysis represented less than 10% of the transfer activity. Even at concentrations far from the K_m, such as 0.1 mM, there was still a 40% transfer (Fig. 6) showing that the activity of Bgt2p was a true transferase activity.

Phenotype of Afbgt2 and Afbgt1/Afbgt2 Mutants—Deletion of the Af*BGT2* and Af*BGT1* genes in the Af*bgt2* mutant were undertaken, as shown in supplemental Fig. 1. PCR and Southern blot analysis confirmed replacement of the Af*BGT2* gene by the *HPH* gene at the gene locus. Also, PCR and Southern blot analysis confirmed gene replacement of the Af*BGT1* locus by the *BLE* gene at the gene locus in the Af*bgt2* mutant (supplemental Fig. S1).

Phenotypic analysis revealed that Af*bgt2* and Af*bgt1*/Af*bgt2* mutants did not display a phenotype different from the wild type strain. Mycelium morphology and germination rate were the same for both strains. Growth rates on different medium (RPMI, Sabouraud, 1% yeast extract + 3% glucose, minimal medium) and at different temperatures (20, 30, 37, and 50 °C) were identical for all strains. Growth on solid Sabouraud medium with increasing concentrations of sorbitol up to 2 M or at different pH values (pH 5, 7, and 9) were similar for both wild type and the 2 mutant strains. After 48 h at 37 °C of growth in the presence of different inhibitors (0.5 mg/ml of Congo red, 1 mg/ml of calcofluor white, 0.6 μg/ml of caspofungin, 0.06 μg/ml of micafungin, 0.6 mg/ml of SDS) MIC values for each molecule were the same for the wild type and mutant strains (data not shown).

Cell wall analysis of both mutants and wild type strain showed no difference in terms of hexose and hexosamine content (data not shown). The branching level of β(1–3)-glucan from both strains was measured by laminarinase A digestion of the alkali-insoluble fraction and HPAEC analysis. The profiles were the same for wild type and mutants with a branched signal corresponding to 5% of the released glucose and laminaribiose (data not shown), showing that branching of the cell wall β(1–3)-glucans was not modified by Af*BGT1*/Af*BGT2* deletion.

DISCUSSION

We report the biochemical and molecular analysis of the first fungal β(1–3)-glucanosyltransferase with a β(1–6)-branching activity, AfBgt2p. Bgt2p cleaves laminaribiose from the reducing end of a β(1–3)-glucan chain and transfers the remaining part mainly inside another β(1–3)-glucan chain by a β(1–6)-glucan linkage.

Orthologous proteins, AfBgt1p in *A. fumigatus* and ScBgl2p in yeast, have also transglycosidase activity. The first enzymatic reaction step is the same for all of these enzymes with the release of the laminaribiose from the reducing end of the donor substrate. However, in contrast to AfBgt2p, the transfer is exclusively performed on the non-reducing end of the acceptor substrate during the second step of the enzymatic reaction. The reasons for having a transfer of β(1–3)-glucan both at the non-reducing end of the donor β(1–3)-glucan and inside the chain are unknown. It could only indicate that the environments (substrate and physicochemical conditions) are not adequate to permit enzymatic reactions *in vitro* that would totally mimic the activity of this protein *in vivo* or to an intrinsic property of the enzyme conformation. Sequence analysis showed that AfBgt1p and AfBgt2p belong to the GH17 family. Based on enzymatic, sequence, and crystallography data, this family contains β-glucanase and transglucosidases (36). The active site of the GH17 family enzyme consists of two glutamate residues acting, respectively, as proton donor and nucleophil residues to cleave β-linked substrates at equatorial bonds and retaining the β-anomeric configuration. The transglycosidase activity of members of the GH17 family has only been demonstrated in the fungal kingdom. In bacteria, proteins belonging to the GH17 family are involved in biosynthesis of cyclic β(1–3),β(1–6)-glu-

cans from *Bradyrhizobium japonicum* (37), but no enzyme activity has been characterized yet. Members of the GH17 family in plant are only endoglucanases cleaving homogeneous $\beta(1-3)$ or heterogeneous $\beta(1-3;1-4)$ linear glucans. Both enzymes have a similar 8-fold β/α barrel three-dimensional structure and substrate specificities are associated to minor differences in amino acid residues (35, 38). In contrast to transglucosidase, β-glucanase does not recognize the reducing end of the β-glucan chain to cleave it, suggesting differences of substrate recognition between endoglucanase and transglycosidase activities belonging to the GH17 family.

A specific comparative genomic analysis of the GH17 family in fungi has been undertaken by BlastP. As shown in Fig. 7, fungal members of the GH17 family can be assigned to five distinct groups: group I including AfBgt1p (AFUA_1G11460) and ScBgl2p, the group II including AfBgt2p (AFUA_3G00270), groups III and IV including the Scw4p, Scw10p, and Scw11p of *S. cerevisiae* and their homologs in *A. fumigatus* AfScw4p (AFUA_6G12380), Scw11p (AFUA_8G05610), and group V composed only from filamentous fungus proteins including proteins encoded by AFUA_5G08780 from *A. fumigatus*. The transglucosidase activity has been identified in groups I and II and it is interesting to note that the differences of transglucosidase activity are correlated with distinct groups. No enzymatic activity has been described for any members of groups III–V. In *S. cerevisiae*, the phenotypic analysis of strains lacking *SCW4* and *SCW10* showed an increase in osmotic instability and in sensitivity to the dye interfering with cell wall polysaccharides that was associated to a higher amount of chitin and alkali-soluble glucans, suggesting that three proteins have a role in cell wall morphogenesis (39, 40).

The double Af*bgt1*/Af*bgt2* mutant has a wild type phenotype. This result suggested that at least one other branching activity is present in *A. fumigatus*. Other members of the GH17 family (AfScw4p and AfScw11p) are potential candidates for this new transglycosidase activity. This hypothesis is currently investigated. The biochemical methodology developed to identify branched $\beta(1-3)$-glucans and the double Af*bgt1*/Af*bgt2* mutant will be essential tools to search for a new $\beta(1-3)$-glucan branching enzyme.

REFERENCES

1. Latgé, J. P. (2007) *Mol. Microbiol.* **66,** 279–290
2. Fontaine, T., Simenel, C., Dubreucq, G., Adam, O., Delepierre, M., Lemoine, J., Vorgias, C. E., Diaquin, M., and Latgé, J. P. (2000) *J. Biol. Chem.* **275,** 27594–27607
3. Beauvais, A., Drake, R., Ng, K., Diaquin, M., and Latgé, J. P. (1993) *J. Gen. Microbiol.* **139,** 3071–3078
4. Hartland, R. P., Fontaine, T., Debeaupuis, J. P., Simenel, C., Delepierre, M., and Latgé, J. P. (1996) *J. Biol. Chem.* **271,** 26843–26849
5. Popolo, L., and Vai, M. (1999) *Biochim. Biophys. Acta* **1426,** 385–400
6. Hurtado-Guerrero, R., Schüttelkopf, A. W., Mouyna, I., Ibrahim, A. F., Shepherd, S., Fontaine, T., Latgé, J. P., and van Aalten, D. M. (2009) *J. Biol. Chem.* **284,** 8461–8469
7. Mouyna, I., Monod, M., Fontaine, T., Henrissat, B., Léchenne, B., and Latgé, J. P. (2000) *Biochem. J.* **347,** 741–747
8. Mouyna, I., Fontaine, T., Vai, M., Monod, M., Fonzi, W. A., Diaquin, M., Popolo, L., Hartland, R. P., and Latgé, J. P. (2000) *J. Biol. Chem.* **275,** 14882–14889
9. Mouyna, I., Morelle, W., Vai, M., Monod, M., Léchenne, B., Fontaine, T., Beauvais, A., Sarfati, J., Prévost, M. C., Henry, C., and Latgé, J. P. (2005) *Mol. Microbiol.* **56,** 1675–1688
10. Goldman, R. C., Sullivan, P. A., Zakula, D., and Capobianco, J. O. (1995) *Eur. J. Biochem.* **227,** 372–378
11. Mouyna, I., Hartland, R. P., Fontaine, T., Diaquin, M., Simenel, C., Delepierre, M., Henrissat, B., and Latgé, J. P. (1998) *Microbiology* **144,** 3171–3180
12. da Silva Ferreira, M. E., Kress, M. R., Savoldi, M., Goldman, M. H., Härtl, A., Heinekamp, T., Brakhage, A. A., and Goldman, G. H. (2006) *Eukaryot. Cell* **5,** 207–211
13. Cove, D. J. (1966) *Biochim. Biophys. Acta* **13,** 51–56
14. Zverlov, V. V., Volkov, I. Y., Velikodvorskaya, T. V., and Schwarz, W. H. (1997) *Microbiology* **143,** 1701–1708
15. Laemmli, U. K. (1970) *Nature* **227,** 680–685
16. Stynen, D., Goris, A., Sarfati, J., and Latgé, J. P. (1995) *J. Clin. Microbiol.* **33,** 497–500
17. Desai, N. N., Allen, A. K., and Neuberger, A. (1983) *Biochem. J.* **211,** 273–276
18. Beauvais, A., Monod, M., Debeaupuis, J. P., Diaquin, M., Kobayashi, H., and Latgé, J. P. (1997) *J. Biol. Chem.* **272,** 6238–6244
19. Martín-Cuadrado, A. B., Encinar del Dedo, J., de Medina-Redondo, M., Fontaine, T., del Rey, F., Latgé, J. P., and Vázquez de Aldana, C. R. (2008) *Mol. Microbiol.* **69,** 188–200
20. Ciucanu, I., and Kerek, F. (1984) *Carbohydr. Res.* **131,** 209–217
21. Fontaine, T., Lamarre, C., Simenel, C., Lambou, K., Coddeville, B., Delepierre, M., and Latgé, J. P. (2009) *Carbohydr. Res.* **344,** 1960–1967
22. Aimanianda, V., Clavaud, C., Simenel, C., Fontaine, T., Delepierre, M., and Latgé, J. P. (2009) *J. Biol. Chem.* **284,** 13401–13412
23. Wishart, D. S., Bigam, C. G., Yao, J., Abildgaard, F., Dyson, H. J., Oldfield, E., Markley, J. L., and Sykes, B. D. (1995) *J. Biomol. NMR* **6,** 135–140
24. Kessler, H., Griesinger, C., Kerssebaum, R., Wagner, K., and Ernst, R. R. (1987) *J. Am. Chem. Soc.* **109,** 607–609
25. Willker, W., Leibfritz, D., Kerssebaum, R., and Bermel, W. (1993) *Magn. Reson.* **31,** 287–292
26. Lamarre, C., Ibrahim-Granet, O., Du, C., Calderone, R., and Latgé, J. P. (2007) *Fungal Genet. Biol.* **44,** 682–690
27. Punt, P. J., Oliver, R. P., Dingemanse, M. A., Pouwels, P. H., and van den Hondel, C. A. (1987) *Gene* **56,** 117–124
28. Mattern, I. E., Punt, P. J., and Van den Hondel, C. A. M. J. J. (1988) *Fungal Genet. Newslett.* **35,** 25
29. Sanchez, O., and Aguirre, J. (1996) *Fungal Genet. Newslett.* **43,** 48–51
30. Girardin, H., Latgé, J. P., Srikantha, T., Morrow, B., and Soll, D. R. (1993) *J. Clin. Microbiol.* **31,** 1547–1554
31. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956) *Anal. Chem.* **28,** 350–356
32. Johnson, A. R. (1971) *Anal. Biochem.* **44,** 628–635
33. Sawaredker, J. S., Sloneker, J. H., and Jeanes, A. (1965) *Anal. Biochem.* **37,** 1602–1604
34. Leitao, E. A., Bittencourt, V. C., Haido, R. M., Valente, A. P., Peter-Katalinic, J., Letzel, M., de Souza, L. M., and Barreto-Bergter, E. (2003) *Glycobiology* **13,** 681–692
35. Varghese, J. N., Garrett, T. P., Colman, P. M., Chen, L., Høj, P. B., and Fincher, G. B. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91,** 2785–2789
36. Henrissat, B., and Davies, G. (1997) *Curr. Opin. Struct. Biol.* **7,** 637–644
37. Bhagwat, A. A., Gross, K. C., Tully, R. E., and Keister, D. L. (1996) *J. Bacteriol.* **178,** 4635–4642
38. Receveur-Bréchot, V., Czjzek, M., Barre, A., Roussel, A., Peumans, W. J., Van Damme, E. J., and Rougé, P. (2006) *Proteins* **63,** 235–242
39. Teparić, R., Stuparević, I., and Mrsa, V. (2007) *Yeast* **24,** 259–266
40. Sestak, S., Hagen, I., Tanner, W., and Strahl, S. (2004) *Microbiology* **150,** 3197–3208